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Narrow Substrate Niche of *Cheilanthes lanosa*, the Hairy Lip Fern, is Determined by Carbohydrate and Lipid Contents in Gametophytes

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ABSTRACT.—*Cheilanthes lanosa* is a xerophytic fern that inhabits rock cliffs and crevices. Morphological features such as cuticle and trichomes assist sporophyte survival. However, the gametophyte stage appears to lack any water-saving features. Previous studies suggest that the gametophyte may balance its water through carbohydrate production and a strong internal osmotic gradient. To investigate the basis for gametophyte survival, lipids and carbohydrates were quantified using the vanillin and anthrone assays. Results suggest that lipids and carbohydrates increase in percent of total biomass (w/w) throughout development. In addition, lipid and carbohydrate content can change with varying culture conditions. Young gametophytes, with high carbohydrate and low lipid content, are relegated to substrates with a potential for a small but continuous water source.

KEY WORDS.—*Cheilanthes lanosa*, gametophyte physiology, water balance

Cheilanthes, the Rock Ferns, is a genus of xerophytic ferns that is generally restricted to rocky habitats (Mickel, 1979; Yatskievych, 1999). *Cheilanthes* sporophytes have evolved morphological characteristics and reproductive mechanisms that aid survival in dry climates and could potentially survive in numerous xeric habitats. For example, *Cheilanthes* sporophytes produce numerous trichomes that help to prevent water loss (Quirk and Chambers, 1981). They produce a thick cuticle, and several species appear bluish-green as a result of this production (Cobb *et al.*, 2005). In addition, *Cheilanthes* species exhibit reduced surface area to volume of leaf cells (Gratani *et al.*, 1998; Hevly, 1963; Pickett, 1931). Thus, there is a relatively small amount of surface area for water loss. Another characteristic that makes these ferns very hardy is their ability to desiccate and still maintain viability. Like many desiccation-tolerant ferns, *Cheilanthes* sporophytes can dry and rehydrate (Quirk and Chambers, 1981). These ferns also engage in mycorrhizal relationships (Palmieri and Swatzell, 2004). Mycorrhizae typically aid in water and nutrient uptake in exchange for photosynthates (Al-Karaki, 1998; Harley and Smith, 1983).

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Survival for ferns, however, also includes reproduction, and sexually reproducing ferns generally require at least a film of water for male gametes to reach the female gametophyte structures, the archegonia (Raven *et al.*, 2005). Some *Cheilanthes* species circumvent this need for water through apogamy (Hevly, 1963; Steil, 1933; Whittier, 1965). Thus, *Cheilanthes* sporophytes possess a multitude of characteristics that enable these ferns to persist through the diploid portion of their life cycles.

Gametophytes, however, lack the majority of these features. They do possess a lipid-based substance that appeared to be exuded by wax glands (Lingle *et al.*, 2004). In addition, the gametophytes can desiccate fully, revive, and produce new gametophytes. Under a slow drying regime, gametophytes may abandon peripheral, vulnerable cells and dry to a bright green, almost crystalline group of cells. These cells can revive when rehydrated and produce new filaments and prothalli (Diamond and Swatzell, 2003). However, these gametophytes are haploid, and only one cell thick (Steil, 1939). Therefore, it is still unclear how the gametophytes, which appear as highly vacuolate, unprotected cells in a single layer, can survive without desiccation or even through it.

Clearly, there must be some physiological mechanism, or combination of mechanisms, that aids in gametophyte survival, but the nature of this mechanism has not been elucidated. The obvious candidate would be a physiological mechanism that can control water uptake and prevent water loss. Previous research has shown that the protonema of at least some *Cheilanthes* species have specific water requirements to germinate (Nondorf *et al.*, 2003) and remain viable as they mature. For example, slender lip ferns germinate and survive optimally on limestone substrate with a specific retention of 20–30 $\mu l \cdot cm^{-3}$ (Dooley and Swatzell, 2002; Nondorf *et al.*, 2003). Studies also show that hairy lip fern gametophytes manage water in their cells through control of aquaporin-like proteins, or water-specific plasma membrane channels (Diamond, 2007). Still, control of aquaporins alone cannot maintain water balance in a desiccating environment. Osmosis, water diffusion across a membrane, although slow, does occur and would place vulnerable gametophyte cells at risk. In hairy lip fern gametophytes, aquaporin-like proteins appear to increase in quantity throughout development (Ricks and Swatzell, 2006), as do lipids and carbohydrates (Diamond *et al.*, 2003, Lingle *et al.*, 2004). Previous studies have shown that gametophytes that are grown in conditions closely resembling the fern's natural environment have higher levels of mono- and disaccharides than gametophytes or protonemal callus grown on agar medium (Abney, 2004). Diamond *et al.* (2003) showed that the total osmotic potential in gametophytes grown under natural conditions is five times that of agar-grown gametophytes. Therefore, it is possible that increased lipid concentrations that block osmosis, increased carbohydrate concentrations that promote water uptake and inhibit water loss, and increased aquaporin-like proteins and/or control of water flow may constitute the survival mechanism for hairy lip fern gametophytes.

To determine if lipids and carbohydrates play a pivotal role in the water balance mechanism of *Cheilanthes* gametophytes, the developmental stages of *Cheilanthes lanosa* (Michx.) D.C. Eat. gametophytes were examined for changes in total lipid and carbohydrate contents. We predicted that as gametophytes mature, lipids and carbohydrates would increase in concentration during their development. We also predicted that the environment would be an influence on lipid and carbohydrate production, with the mature gametophytes grown on dry sand substrate exhibiting a higher level of both lipids and carbohydrates.

METHODS

Plant Collection

Hairy lip fern sporophylls were collected in the fall after the first frost from a sandstone bluff 0.25 mi north of Makanda, Illinois, placed in glass 9 cm-diameter Petri dishes, and stored in the dark at 4°C. After several months, sporophylls were crushed using a mortar and pestle. *Cheilanthes lanosa* spores average 40 μm in diameter (Devi *et al.*, 1970) and spores were separated from the plant material using a 65 μm brass mesh sieve. Spores were stored at 4°C in the dark.

Plant Growth and Materials

Wet grown (WG) gametophytes and protonemal callus.—Spores were surface sterilized in a 7% (v/v) commercial bleach solution with 0.1% (v/v) Triton X-100 for 10 min. Spores were then rinsed in sterile ddH_20 and sown on a modified (Smith, 1992) tissue culture medium (TCM) in 9 cm-diameter disposable, sterile Petri dishes. Spores were incubated at 25°C in 0.175 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of continuous far red light (650–705 nm) for 10 days.

Following germination and protonemal development, the plates were separated. Some plates were left in the far red light to induce and maintain protonemal callus growth. The remaining plates were then exposed to continuous white light (405–710 nm) and the protonemata began planar growth into gametophytes.

Dry grown (DG) gametophytes.—Spores were sown on fine grain white sand (Décor Sand, Activa Products Inc., Marshall, TX) in 9 cm glass Petri dishes, wetted with 20 ml of TCM, and incubated first in far red light, then white light as the WG cultures described above. However, unlike the sterile cultures of WG and callus, the DG cultures were not sealed. After DG gametophytes reached the prothallus stage, they were wetted erratically upon drying with ddH_2O. DG gametophytes were grown to maturity, that is, until the first sporophytes appeared and wax glands were abundant.

Sampling Procedures

Each sample consisted of clumps of gametophytes, approximately the same size, withdrawn haphazardly from approximately 12 plates of each treatment.

To avoid potential desiccation, samples were placed immediately into preweighed 0.5 ml microcentrifuge tubes and frozen at −80°C. After all samples were gathered, they were removed from storage, but not opened until the wet weight of each had been determined. Thus, any potential water loss within the tube during storage was also taken into account. Following determination of the sample weights, vanillin or anthrone assays were then performed.

Anthrone Assay

Gametophytes (n = 98 for each stage) were collected from agar or sand using needle-tipped tweezers. Gametophytes were placed in 1 ml microcentrifuge tubes, weighed to obtain total sample weight, and stored at −80°C. Gametophytes were then placed in 1.5 ml microcentrifuge tubes and homogenized with a micropestle in 62.5 µl of 0.5 M Na_2SO_4. Following homogenization, 1.25 ml anthrone reagent (Judd, 2006; van Handel, 1985a) was added to the sample and vortexed. Tubes were then heated at 100°C for 12 min, vortexed again, and cooled to room temperature. The absorbance of each sample was measured at 625 nm using a Beckman DU 640B spectrophotometer. Sucrose standards (0–100 µg/ml) were prepared from a 0.1 g/L stock solution and absorbance was measured as above.

Vanillin Assay

Gametophytes (n = 100 for each stage) were collected and weighed as above. Samples were stored in 1.0 ml microcentrifuge tubes at −80°C. Samples were then transferred to 1.5 ml microcentrifuge tubes and homogenized with a micropestle. Following homogenization, 100 µl of a 1:1 chloroform/methanol was added to each sample. The samples were centrifuged at approximately 10,000 rpm for 10 min. The supernatant was retained and allowed to dry overnight. Samples were vortexed and heated at 100°C for 10 min in 200 µl of concentrated H_2SO_4. Samples were cooled to 25°C and vortexed. Samples were then transferred to glass shell vials, mixed with 3 ml of vanillin reagent (Barnes and Blackstock, 1973; van Handel, 1985b) and incubated for 30 min. Absorbance was determined at 525 nm using a Beckman DU 640B spectrophotometer. The lipid standard, almond oil (NowFoods, Bloomingdale, IL), was prepared and measured as above. Stock concentration was 1:1,000 in a 1:1 chloroform/methanol mixture. Average weight (n = 100) of standards was 4.83 µg/ml. Standard dilutions were 1X, 1/2X, and 1/4X. Absorbance was used to calculate lipid weights in samples.

Data Analysis

Statistical differences in percent lipid and carbohydrate content per total weight were determined by analysis of variance (ANOVA) followed by a

Tukey's studentized test using the SAS General Linear Model Procedure (SAS 1999–2000).

Results

Plant Culture

It was important to produce protonema *en masse* without altering the uniseriate, filamentous growth. Following spore germination on TCM, protonema developed into callus under extended red light treatment (Fig. 1A). Blue light wavelengths in white light exposure induced planar growth (Fig. 1B). However, under red light treatment in tissue culture, cells were continuously produced in numerous filaments.

Gametophytes in agar culture with blue light developed normally (Fig. 1C; WG). The protonemal remnant persisted in many of the gametophytes. Gametophytes produced antheridia, but no archegonia, similar to previous reports of *Cheilanthes feei* T. Moore gametophytes in the wild (Steil, 1933). The WG gametophytes resembled dry sand cultured gametophytes in size and morphology (Fig. 1D; DG). However, DG gametophytes on sand generally completed the haploid portion of their life cycle including wax gland and sporophyte production (WG gametophytes did produce wax glands when left long enough in culture so that the agar substrate began to dry from age; this was rare and WG gametophytes never produced sporophytes; Fig. 2).

Anthrone Assay

Calculation of the means (Table 1) from the anthrone assay revealed that the mean percentage of carbohydrates per total weight was 23.53% ±15.21% for the WG treatment, 12.21% ±30.29% for the DG treatment, and 17.31% ± 9.73% for the protonemal callus treatment (Fig. 3). Analysis of variance (ANOVA) followed by a Tukey's studentized test revealed that there was a significant difference in percent carbohydrates between the WG and DG treatments, but there was no significant difference between protonemal callus and the other treatments.

Vanillin Assay

Lipid concentration in callus, WG, and DG gametophytes was assessed using a vanillin assay. Assay results show a mean percent lipid concentration of 0.72% ± 0.46% for the WG treatment, 0.22% ± 0.20% for the DG treatment, and 0.10% ± 0.14% for the protonemal callus treatment (Fig. 4; Table 1). An ANOVA followed by a Tukey's studentized test revealed that there was a significant difference between each treatment.

Overall, the percent carbohydrates of total weight were much higher in all three treatments than the percent lipids of total weight. Means of lipid concentrations per total weight ranged below 1.0%. However, carbohydrate concentrations were as much as 23.0% of total weights.

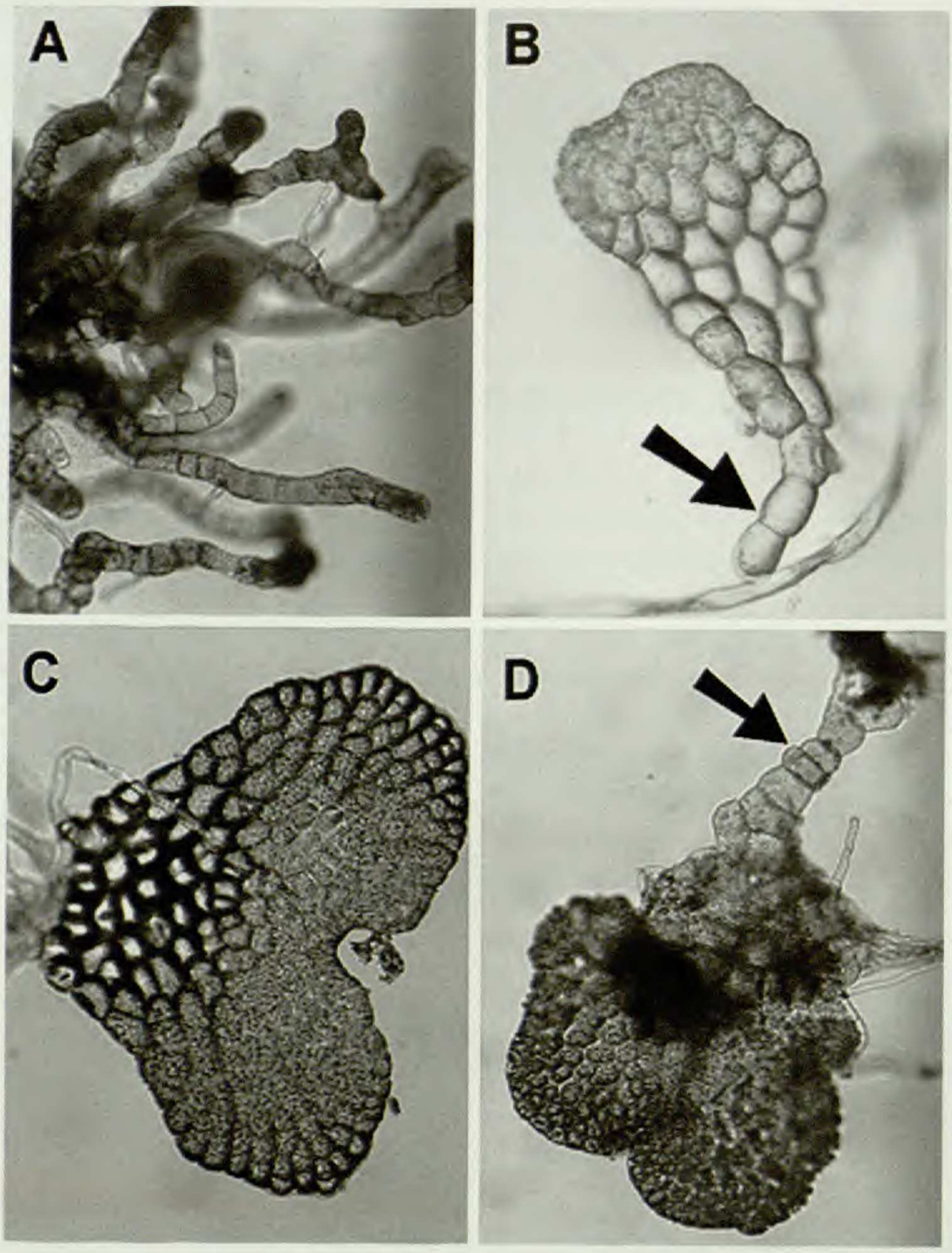

FIG. 1. Cultured Gametophytes of *Cheilanthes lanosa*. Gametophytes in agar culture underwent normal development from germination, through the protonemal stage **(A)**, to prothallus development **(B)**. Mature gametophytes on agar **(C)** and in dry culture **(D)** still bear the remnant of the protonemal stage **(arrow)**. Mature gametophytes produced antheridia.

DISCUSSION

Effects of Environment and Development on Lipid and Carbohydrate Production

Previous research on hairy lip fern gametophytes revealed an increased ability to manage water balance as gametophytes mature (Diamond *et al.*, 2003; Diamond, 2007). Therefore, we hypothesized that carbohydrate and lipid concentrations would also increase through the gametophyte generation with maturity. Lipids in cuticle materials or intracellular lipids that line cell

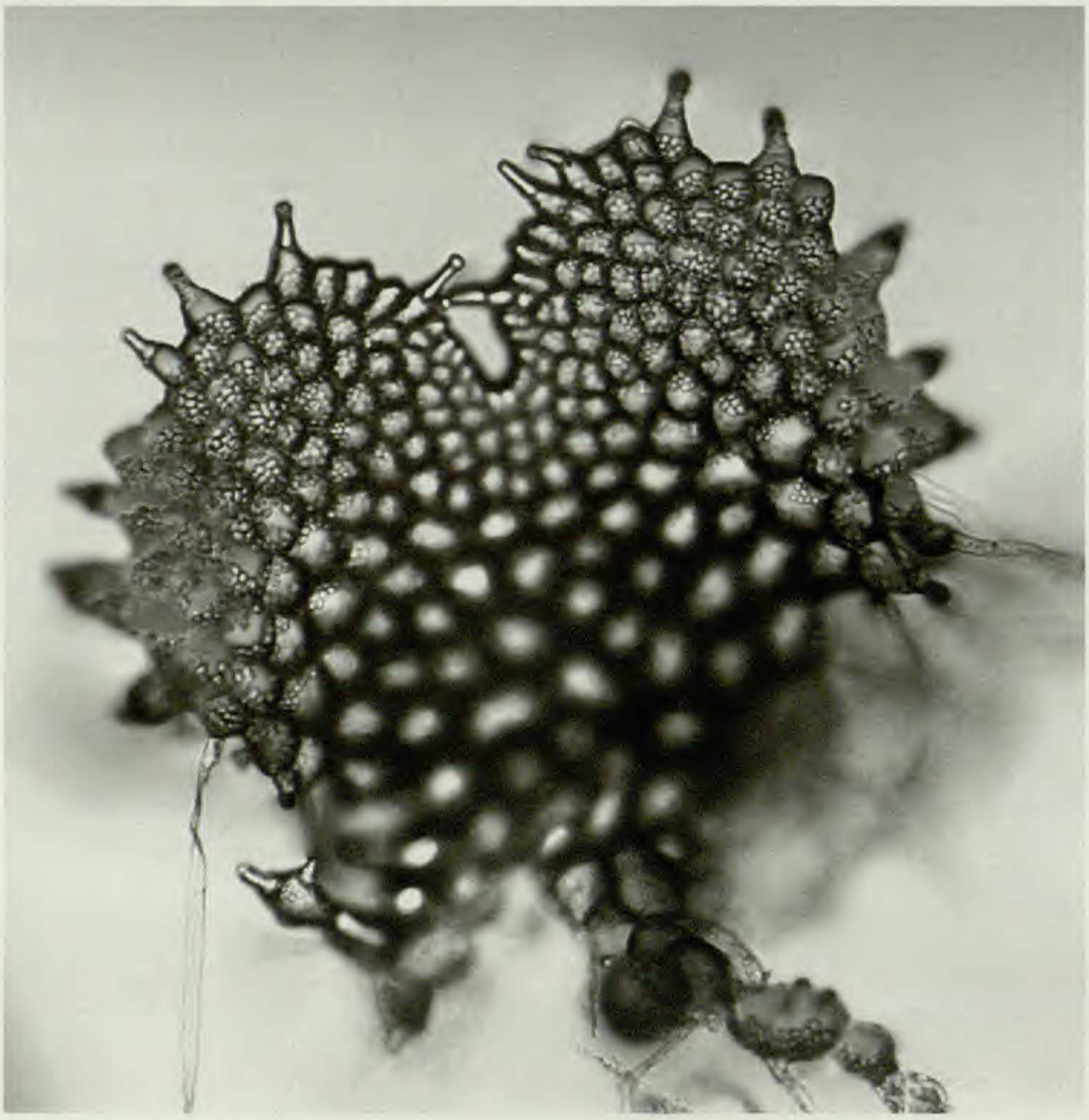

FIG. 2. Mature gametophytes of *Cheilanthes lanosa* grown under dry conditions (DG). Wax glands are commonly produced on cell surfaces. Prior to forming a sporophyte, which will draw resources from the gametophyte, a thickened sporophyte pad will develop in the center of the gametophyte.

peripheries could potentially block osmosis, and carbohydrates, utilized as an internal osmotic gradient, could increase water uptake and inhibit water loss (Lingle *et al.*, 2004; Schneider *et al.*, 2003). We predicted that normal gametophyte development would include an increase in lipids and carbohydrates that could protect the gametophyte from desiccation and prepare it to manage water in an unpredictable environment. This prediction was only partially accurate. Gametophytes grown in a consistent environment, such as the wet agar-grown gametophytes (the protonematal callus and the WG gametophytes) steadily increased in lipids and carbohydrates. However, we did not predict that gametophytes grown in substantially dryer, unpredictable conditions (DG) would be significantly lower in percent lipid and carbohydrate content than their mature wet grown counterparts or that the samples would vary so widely. Instead, the results of this study suggest that carbohydrate and lipid concentrations in different gametophyte stages could be the result of both environmental and developmental factors.

The wet agar-grown cultures showed a pattern of increase in both carbohydrate and lipid concentrations from protonemal callus to mature

TABLE 1. Mean % carbohydrate and lipid concentration in gametophyte culture. Analysis of variance (ANOVA) followed by a Tukey's studentized test ($P<0.05$) showed a significant difference between percent carbohydrates in wet-grown (WG) and dry-grown (DG) treatments. There was no significant difference between percent carbohydrates in callus and other treatments. ANOVA followed by a Tukey's studentized test revealed a significant difference between each treatment. Overall, gametophytes contained far more carbohydrates than lipids.

Gametophyte Culture	% Carbohydrate (n=98)	% Lipids (n=100)
Callus	17.3±9.7	0.1±0.1
WG	23.5±15.2	0.7±0.5
DG	12.2±30.3	0.2±0.2

gametophytes (WG). Protonemal callus contained 17.31% carbohydrates per total weight and this level rose to 23.53% upon maturity. In an arid environment, a strong internal carbohydrate concentration would allow gametophytes to maintain a strong internal osmotic gradient. The gametophytes can control whether or not water can flow through their plasma membrane aquaporins (Diamond, 2007), so that a gametophyte could draw water hygroscopically in a humid atmosphere. However, this steady increase occurred only as the gametophytes matured in a protected environment and this osmotic gradient was able to develop without depletion or risk of

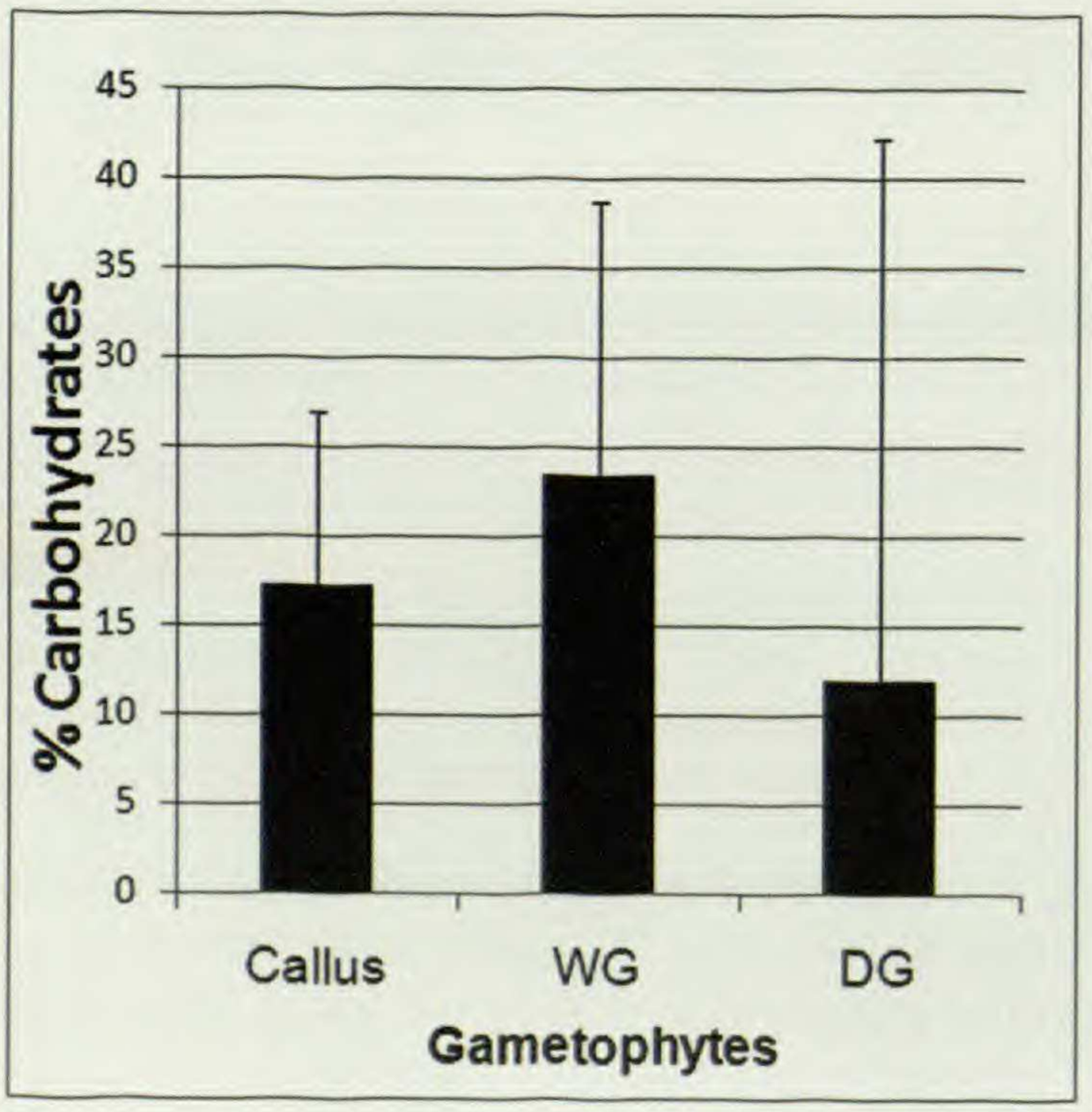

FIG. 3. Mean (n = 98) carbohydrates content per total weight in gametophytes of *Cheilanthes lanosa*. There was no statistical difference ($P < 0.05$) between callus and WG samples. There was also no significant difference between callus and DG samples. There was a statistical difference between the DG and WG samples. Solid = growth on wet agar-based nutrient medium. Stripe = growth on fine sand with erratic watering. WG = wet-grown gametophytes. DG = dry-grown gametophytes.

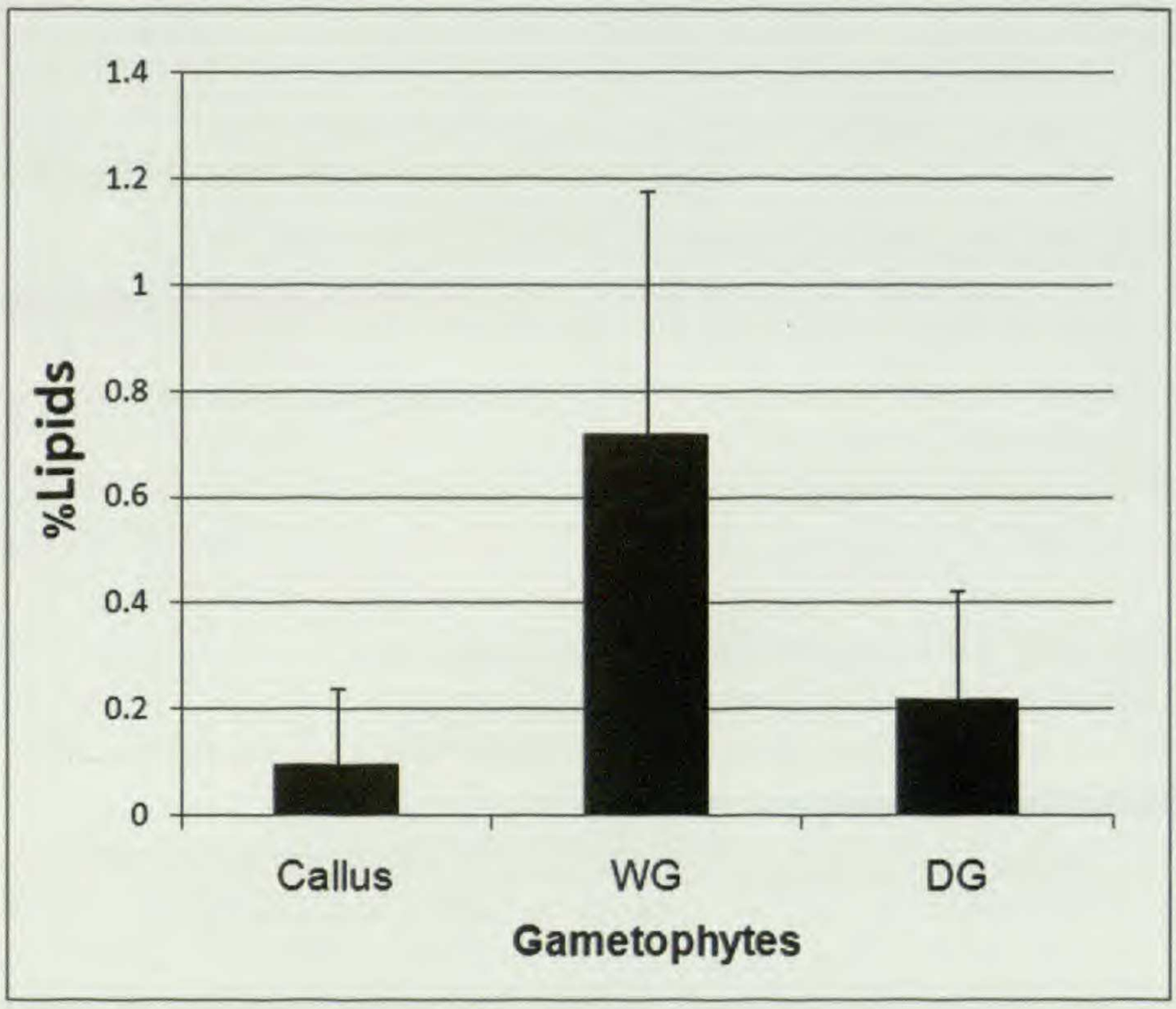

FIG. 4. Mean percent (n = 100) lipids per total volume weight of callus, wet grown (WG), and dry grown (DG) gametophytes. There was a statistical difference ($P < 0.05$) among all sample means. Solid = growth on wet agar-based nutrient medium. Stripe = growth on fine sand with erratic watering.

desiccation. The large standard deviation in these samples is likely due to the variations in maturity of gametophytes sampled as batches and not as individuals (which would have been very difficult). Thus, sample collection, in which each sample was a clutch of individuals grasped from one of a dozen plates with tweezers, and collected at a precise time and date, would naturally introduce variation. Still, the standard deviation was consistent between the WG and callus stages. This suggests a constant range of response within the wet agar environments.

DG gametophytes exhibited a much lower concentration of carbohydrates and lipids than expected. These gametophytes produce lipid-based exudates from trichome-like structures, and these structures appear to be similar in morphology to wax glands of related species which are also xerophytic (Diamond *et al.* 2003; Lingle *et al.* 2004). Though the trichome-like structures in hairy lip ferns have not been fully characterized microscopically, it is likely that they are also wax glands. Trichomes produced a halo of lipid-based exudates and this exudate appears to cover the entire gametophyte surface (Lingle *et al.*, 2004). Thus, a high concentration of lipids in the DG gametophytes was expected. In addition, other xerophytes can mobilize lipids to the cell periphery of vessel elements and thereby block transpiration (Schneider *et al.*, 2003). However, *Cheilanthes lanosa* gametophytes do not possess vascular tissue, but are single celled in thickness. Lipid localization

was beyond the scope of this study, but regardless of localization, all lipids would still be included in the total percentage of tissue mass. With respect to carbohydrate concentrations and because DG gametophytes are impervious to high solute perturbation (up to 500 mM NaCl for 1 hr; Diamond *et al.*, 2003), we predicted a high concentration of carbohydrates as well.

The lower concentration of lipids and carbohydrates found in the DG gametophytes in this experiment could be due to several factors. WG gametophytes and protonemal callus were grown in an environment with consistent water and nutrient availability, and their resources may have been committed to growth and development. Alternatively, DG gametophytes may have utilized their resources differently, perhaps for stress responses and secondary metabolic pathways. The high variation in the DG treatments is characteristic of a plant population exhibiting a high level of signaling activity and response in a stressful environment (Fitter and Hay, 1987; Taiz and Zeiger, 2006). Although WG gametophytes rarely complete their life cycle to sporophyte formation, but dry down with the agar and slowly produce wax glands, DG gametophytes consistently produce sporophytes in culture (Diamond and Swatzell, 2003). At the time the DG gametophytes were collected, they were poised to complete the haploid stage and begin sporophyte production through apogamy. Because sporophytes are nutritionally dependent on gametophytes, it is possible that the gametophytes invested their resources for cell division machinery and proteins (or for other cell components that were not measured in this study) instead of starch storage or cytoplasmic sugars. For example, Abney (2004) showed using HPLC that dry-grown gametophytes contained a higher soluble concentration of mono- and disaccharides than wet-grown gametophytes or protonemal callus. Mono- and disaccharides were immeasurable in protonemal callus (Abney, 2004). In addition, Diamond *et al.* (2003) showed that total osmotic potential in DG gametophytes is five times that of WG gametophytes. Taken together with the results of this study, this suggests that a portion of the osmotic gradient in DG gametophytes has still not been described.

Another explanation of the differences between the two types of mature gametophytes could be the differences between lipid and carbohydrate extraction methods and the nature of the cell wall and cuticle. In the anthrone assay (Judd, 2006; van Handel, 1985a), all cell components were liberated with H_2SO_4. However, in the vanillin assay (Judd, 2006; van Handel, 1985b), sample preparation involved a lipid extraction from the cell wall surfaces. Following extraction and solubilization, cell wall materials were pelleted and discarded. Lipids still bound to and within the cuticular layer of the cell wall, if a cuticle was actually present, would have been discarded. Thus, lipids in DG gametophytes that were committed to cuticle production would have been discarded and not measured in the assay. Still, there is no evidence yet whether the DG gametophytes actually produce cuticle, or whether they simply produce lipid substances via wax glands. Regardless of the basis for the differences between dry grown and wet agar-grown stages, it appears that the

environment is as much of a factor in lipid and carbohydrate production as is development.

Changes in Development Leave Protonema Vulnerable

Development may also be responsible for the establishment of a strong, internal osmotic gradient within gametophytes. Mature DG and WG gametophytes have higher concentrations of carbohydrates and lipids than the respective protonema. Gametophytes appear to be developmentally programmed to increase the concentration of these molecules as they mature. The high concentration of carbohydrates and lipids allows mature gametophytes to remain impervious to desiccation. The limiting stage for hairy lip fern gametophytes appears to be the protonematal stage. When a hairy lip fern spore germinates, the first few cells emerge as uniseriate, filamentous protonema (Raghavan, 1980, 1989). There appears to be no cuticle on these cells, which must grow and divide rapidly as the prothallus develops. Throughout the gametophyte stage, water uptake is controlled through aquaporin-like proteins; plants can potentially control aquaporin function based on a blue-light responsive phosphorylation switch (Johansson *et al.*, 1996; Johnson and Chrispeels, 1991; Kaldenhoff *et al.*, 1995). Thus, protonemata are able to close their aquaporins when stressed, but this does not make them impervious to desiccation (Diamond, 2007). Eventually, because the protonema has very few lipids and no lipid-producing trichomes, osmosis across the plasma membrane and cell wall will lead to desiccation. Conversely, if too much water is present, the water eventually moves by osmosis, in spite of aquaporin control, across membranes into protonemal cells in high amounts, compromising the protonema (Nondorf *et al.*, 2003; Diamond, 2007). Overall, data suggest that the high internal osmotic gradient that drives rapid water uptake through aquaporin-like proteins is also a factor limiting the protonemal stage. Consequently, they must live in an environment that contains the right amount of water (Dooley and Swatzell, 2002).

Hairy lip ferns are primarily found on sedimentary rocks, in which there is a small amount of water continuously present. Because the protonemata is limited by the lack of cuticle, a low lipid content, and a high carbohydrate osmotic gradient, it is also limited to environments that offer a constant, low level of moisture. The high internal osmotic gradient that drives rapid water uptake through aquaporin-like proteins for prothallus production is also the factor that limits the protonemal stage. These ferns are typically relegated to sedimentary substrate by the narrow conditions under which the protonema can live.

Conclusion

Overall, data from this study suggest that hairy lip ferns increase carbohydrates and lipids as a normal process of development. However, there is also a difference in lipid and carbohydrate content between mature

gametophytes on agar and on dry sand, and environmental factors also play a role. DG gametophytes, which consistently develop into sporophytes, contain fewer carbohydrates and lipids than those grown on wet agar. Interestingly, although the mature gametophytes are fairly impervious to desiccation because of their high internal osmotic gradients and lipid content, protonemata are vulnerable and limited. Because they contain a high internal osmotic gradient but lack external lipid protection, protonema can readily become hypotonic when exposed to the water required for quick prothallus development. Therefore, although they enjoy a large range throughout the eastern United States, hairy lip fern gametophytes are typically limited to a narrow niche of sedimentary rock.

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